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ORIGINAL PAPER

Plasmids for expression of heterologous proteins in *Rhizopus oryzae*

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Abstract *Rhizopus oryzae* has long been used for enzyme production (e.g., glucoamylase and lipase), organic acid synthesis, and various fermented food applications. In this work, we describe a set of plasmid-based expression vectors that can be used for the production of heterologous proteins in *R. oryzae*. Three plasmid vectors have been created using either the glucoamylase A (*amyA*), pyruvate decarboxylase (*pdcA*), or phosphoglycerate kinase (*pgk1*) promoters to drive expression of heterologous proteins. All three plasmids use the *pdcA* terminator for transcription termination, the *pyrG* gene for restoration of uracil prototrophy, and an ampicillin resistance gene and origin of replication for maintenance in *Escherichia coli*. We have expressed green fluorescent protein (GFP) and compared transcription and protein accumulation for each of the expression vectors. Accumulation of GFP transcript and protein was directly correlated with the choice of promoter with *pdcA* > *amyA* > *pgk1*. Transcript level appears to parallel GFP protein accumulation. Plasmid copy number had little impact on transcription or protein accumulation. These vectors should be useful for overexpression of heterologous proteins and potentially, metabolic engineering of *Rhizopus* strains.

Keywords *Rhizopus oryzae* · Green fluorescent protein · Heterologous protein expression · Gene copy number · RT-PCR

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Introduction

Considerable effort in heterologous protein expression technologies using modified promoters has focused on enzyme production, which is currently estimated to have annual sales in excess of $1 billion. Much of this effort centers on tailoring microorganisms to produce desired enzymes required for conversions in applied industries (e.g., textiles, biomass conversion), as well as animal feed and food/beverage-processing facilities. Industrial production of hydrolytic enzymes, such as cellulase, amylase, protease, and lipase, has long been the domain of the filamentous fungi as they are robust and adept at secreting large quantities of enzyme. However, currently available fungal expression systems have yet to achieve a reproducible high yield of heterologous proteins and considerable work is still necessary to identify key problem areas in industrially relevant filamentous fungi.

Research into recombinant protein expression by filamentous fungi has largely focused on the Ascomycetes, *Aspergillus* sp. and *Trichoderma*, due to well established techniques for genetic manipulation. Expression systems using modified promoters have also been explored with several Mucorales fungi. The Mucorales fungus are unique in that they normally replicate transformed plasmid DNA autonomously in large multi-copy concatenated structures, rather than single or low-copy integration into the genome, as is typical with most other fungi. Although the large concatenated structures have been shown to be mitotically unstable in the absence of selection, the increased copy number of genes could potentially lead to higher production of protein (Appel et al. 2004), offering a unique opportunity not available in other fungal systems. To date, the most common use of these promoter modifications in *Mucor* (Appel et al. 2004), *Absidia* (Wostemeyer et al. 1987; Burmester 1992), *Phycomyces* (Obraztsova et al. 2004), *Rhizomucor* (Appel et al. 2004), and *Rhizopus* (Yanai et al. 1991) have been for the expression of genes used for selection of transformant strains, based on resistance against specific antimicrobials (e.g. kanamycin, neomycin, and blasticidin S). Promoter studies have also been performed in several of these fungi using heterologous expression of reporter genes, such as green fluorescent protein (Schilde et al. 2001), β-galactosidase (Yanai et al. 1991), and β-glucoronidase (Takaya et al 1994, 1995). More recent efforts to test the Mucorales fungi for production of proteins of interest have centered primarily on *M. circinelloides* (Wolff et al. 2002; Houghton-Larsen and Pedersen 2003; Papp et al. 2006).

Even though results for heterologous protein expression in other Mucorales fungi have been encouraging, very little work in this area has been performed with *R. oryzae*. This fungus is well accepted industrially because of its GRAS status, robustness, ease of use, and capacity to secrete large amounts of enzymes (e.g., glucoamylase, lipase, peptidase). We have now developed a series of expression vectors to test the ability of this organism to produce heterologous proteins using several different promoter modifications. The expression plasmids differ in that each contains a unique promoter sequence to control the timing and level of expression for recombinant proteins. The phosphoglycerate kinase 1, *pgk1*, promoter is generally considered constitutive; the pyruvate decarboxylase, *pdcA*, promoter is expressed primarily in the presence of glucose; and the glucoamylase A, *amyA*, is highly inducible using starch or cellobiose. The upstream regulator regions of the phosphoglycerate kinase and the glucoamylase genes have been used successfully for heterologous protein expression in other Mucorales fungi (Yanai et al. 1991;Takaya et al. 1994, 1995; Houghton-Larsen and Pedersen 2003), while the *pdcA* promoter has not been used previously. All three recombinant plasmids rely on the transcription termination sequence obtained from the *pdcA* gene. The *pyrG* gene is also included on plasmids for selection based on uracil protrophy (Skory 2002). The Green Fluorescent Protein (GFP) variant S65T was chosen to test these vectors, since it has been successfully expressed in a number of filamentous fungi, does not require additional substrates, can be used as a transcriptional or translational fusion, and provides a tool for future study of the *Rhizopus* secretion system and protein localization (Santerre Henricksen et al. 1999; Gordon et al. 2000; Schilde et al. 2001; Lorang et al. 2001).

The goal of this study was to demonstrate functionality of each of the promoter fragments and establish an initial baseline of recombinant protein expression. Providing this foundation is critical for estimating the capacity of *R. oryzae* to express heterologous proteins and allowing future systematic improvements in expression. To this end, we chose to express GFP internally to avoid potential issues related to secretion from the mycelia and proteolytic degradation. Transcriptional profiling of transformants containing the expression constructs was also performed to gain a clearer understanding of transcript formation and stability as little information is available in this regard as it relates to *R. oryzae* and other Mucorales fungi. To determine if gene copy number could have an impact on protein or transcript accumulation, *R. oryzae* was transformed with circular plasmid DNA that is expected to form high copy number concatemers enabling us to determine what, if any, impact gene copy number has on heterologous protein expression. We show in this work that while choice of promoters had significant impact on mRNA transcript levels and protein expression, copy number does not appear to have significant effect. Furthermore, there appears to be a correlation between transcript levels and accumulated protein.

Materials and methods

Strains, media, and transformation

Rhizopus oryzae NRRL395 was the source of DNA for isolation of the *pdcA*, *pgk1*, and *amyA* promoters, as well as the *pdcA* terminator. *Escherichia coli* TOP 10

(Invitrogen, Carlsbad, CA, USA) was used as the host for all cloning procedures and propagation of plasmids. *R. oryzae* strain pyr17 (Skory 2002), a *ura*-strain, was used as the host for recombinant expression. Transformation of *Rhizopus* strain pyr17 was performed by previously described methods using biolistic methods (Skory 2002). Approximately 5–7 days following bombardment, spores were collected and diluted in sterile water to obtain single spore isolates. Only one isolate per plate was used for further analysis to avoid using multiple progeny originating from the same transformation event. Single spore isolates were then plated on minimal RZ media (Skory 2002) and allowed to sporulate. The collected spores were frozen as glycerol (15% w/v) stocks and used to inoculate media for expression studies. Shake flask cultures were performed in 250 ml unbaffled Erlenmeyer flasks containing 50 ml minimal RZ media minus uracil at 30°C and 200 RPM.

Expression vector construction

Construction of the initial expression vector relies on the untranslated promoter and terminator regions of the *R. oryzae pdcA* gene with a previously described genomic clone (Skory 2003), as template for PCR and subsequent cloning. A 1,091-bp *pdcA* promoter region was PCR amplified using *Pfu* Turbo polymerase (Stratagene, La Jolla, CA, USA) with primers pdcProF and pdcProRSph (Table 1) and subsequently cloned into pCR2.1-Topo (Invitrogen). The reverse primer incorporated a transition mutation at the adenosine nucleotide-2 to the ATG start codon in order to incorporate a *Sph*I site. Likewise, an 805-bp *pdcA* terminator region was amplified with primers pdcTerFPac and pdcTerR (Table 1) and also cloned into pCR2.1-Topo. The forward primer for the terminator region was modified with a *Pac*I site, such that there would be no difference in the 3′ untranslated region, if relying on the TAA stop codon in this site. The *pdcA* promoter and terminator fragments were released from the pCR2.1-Topo vector using *Bam*HI/*Not*I and *Not*I/*Sac*I, respectively, and then sequentially ligated into *Bam*HI/*Sac*I sites of pPyr225 (Skory 2002). The resulting 7.23 kb expression vector, pPdcEx, was designed so that heterologous gene inserts could rely on the start and stop codons included in the *Sph*I/*Pac*I sites.

The subsequent *pgk1* and *amyA* expression vectors were obtained by switching the promoter regions in the pPdcEx plasmid. The *pgk1* genes were obtained from a genomic library in this study. This was accomplished by first obtaining an internal region of the *pgk1* gene from *R. oryzae* NRRL 395 DNA using primers pgkF1 and pgkR1 based on the *R. niveus pgk1* sequence. A 1,070-bp PCR product was obtained, sequenced to confirm identity to *pgk1*, and then used as probe to screen a genomic library as previously described (Skory 2000). Two sets of unique overlapping clones were obtained and identified as *pgk1* and *pgk2* based on sequence similarity to the *Rhizopus niveus* genes. These sequences are deposited in GenBank with accession numbers DQ279101 (*pgk1*) and DQ279102 (*pgk2*).

The *pgk1* region chosen to replace the *pdcA* promoter sequence in plasmid pPdcEx was amplified using pgkFXma and pgk3SX (Table 1) containing

Table 1 Primers/probes used for PCR and qRT-PCR

Primer or probe	Sequence (5′ → 3′)
pdcProF	GCTAAAGTTTATCAGCTTCAATCCAT
pdcProRSph	GAAGGCATGCTTTTAAATTTGTTTTGTAGAG
pdcTerFPac	TTAATTAAAATCTTAGAATTCATCTTTTTTTGTATCAT
pdcTerR	ACTCTACCGTCTGCTCTTTTGTCT
pgk F1	GGTCGCCCTAACGGTGAAGC
pgk R1	GATAGCAAGAAAAGGGCGAGAGG
pgkFXma	CCCGGGTCAAAATTCAAAACCGTATCACAT
pgk3SX	TCTAGAGCTTGTTAGATAAAGCATGCTGTTG
amyAProXma	TGGCCCCGGGATTCCATGTCCACTTC
amyAPro3SX	TCTAGAGCATGCTAGAGAAAGACCAAT
MCS-	AGCGGCGCCTACGTAGGCCTCTAGAGCATGC
MCSrev	GGTTAATTAAGCGGCCGCTAGCGGCGCCTACGT
GFPSph	GCGCATGCGAATGAGTAAAGGAG
GFP3Pac	CCTTAATTAATTATTTGTATAGTTCATCC
GFP5Taq3-3	TCTGTCAGTGGAGAGGGTGA
GFP3Taq3	TACATAACCTTCGGGCATGG
GFPprobe3	CCCAGATCATATGAAACGGC
18STaq5	TGGAGTGATTTGTCTGGTTAATTCCGATAA
18STaqAS	CATCTAAGGGCATCACAGACCTGTTATT
18Sprobe	CGGCTTGAAGCCGATAGTCTCTCTAA

engineered *Xma*I and *Sph*I sites, respectively. The 364 bp-product was further modified by adding a multiple cloning site (MCS) downstream of the *Sph*I restriction site containing an available start codon. This was accomplished by sequentially amplifying the promoter fragment with pgkFXma and 3′ primers MCS- and MCSRev (Table 1). The MCS contains the following unique restriction sites: *Sph*I, *Xba*I, *StuI*, *SnaB*I, *Nar*I, *Nhe*I, *Not*I, and *Pac*I. Finally, the *pdcA* promoter was excised from pPdcEx with *Xma*I and *Pac*I and replaced with the *pgk1* promoter fragment with the MCS, resulting in the 6.421 kb plasmid pPgkEx.

The *amyA* expression construct was developed by amplifying an 894 bp *amyA* promoter fragment using primers amyAProXma and amyAPro3SX (Table 1) with genomic DNA as the template. The *pgk1* promoter was removed from pPgkEx using *Xma*I and *Sph*I, maintaining the MCS, and the *amyA* promoter fragment ligated into the vector backbone resulting in the 6.958 kb plasmid pAmyAEx. All constructs were sequenced to confirm the fidelity of the promoter fragments, terminator, and MCS.

Construction of GFP vectors

For construction of the vectors for *gfp* expression, GFP S65T was PCR amplified using primers GFPSph and GFPPac (Table 1) with pVT100U-mtGFP (Westermann and Neupert 2000) as the template. Primers contained engineered *Sph*I and *Pac*I sites in the 5′ and 3′ primers, respectively. The respective expression vectors containing the three different promoters were digested with *Sph*I and *Pac*I, and the *gfp* PCR fragment ligated into the respective vector, resulting in plasmid vectors pPdcA-*gfp*, pAmyA-*gfp* and pPgk1-*gfp*. All constructs were sequenced to confirm the fidelity of the inserted *gfp* gene and splice sites.

Protein expression and assays

Spore preps of individual isolates of each *gfp* expression construct were used to inoculate 50 ml cultures consisting of minimal RZ media and containing 1% (w/v) glucose (Skory 2002) for the pPgk1-*gfp*, pPdcA-*gfp*, and 1% cellobiose (w/v) for the pAmyA-*gfp* isolates. After 72 h, mycelia was collected, placed on a slide, and viewed on an Olympus BX60 microscope with fluorescent detection achieved using a fluoroscein isothiocyanate (FITC) filter set (Olympus America, Inc., Melville, NY, USA). Images were captured with an Olympus DP70 CCD camera (Olympus America, Inc.). Confocal images of a pPdcA-*gfp* transformant were captured from germination through formation of mycelial branching on a Leica DMRXE using an excitation wavelength of 488 nm.

For SDS-PAGE and Western blot experiments, protein was extracted from approximately 10 mg of wet mycelial tissue using 500 µl 50 mM potassium phosphate, pH 7.0 buffer, and 500 µl glass beads in 1.7 ml centrifuge tubes using a FastPrep FP120 instrument (Qbiogene, Carlsbad, CA, USA). The resulting crude protein samples were centrifuged at 10,000×*g* for 10 min to eliminate cellular debris. Protein content of the crude extracts was determined using the Quickstart Bradford Dye Reagent (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) used as the standard. Approximately 20–40 µg of cleared lysate from each of the *R. oryzae* isolates were loaded onto 4–20% SDS-PAGE gels (BioRad) along with an eGFP standard (Clontech, Palo Alto, CA, USA) and Kaliedoscope molecular weight markers (BioRad). The SDS-PAGE gels were run at 100 V until the dye front reached the bottom of the gel. The SDS-PAGE gels were then either stained with Coomassie blue or used for western hybridization analysis. The western blots were performed by standard methods (Towbin 1979) with transfer to a PVDF membrane (Amersham, Piscataway, NJ, USA) and BSA as the blocking reagent. Detection was achieved using a monoclonal eGFP antibody (Clontech) as the primary and an alkaline phosphatase conjugated anti-mouse secondary antibody (Sigma, St Louis, MO, USA) followed by development with BCIP/NBT-Blue liquid substrate (Sigma). Protein quantity was determined by relative densitometry of the western blot samples to a GFP standard of known concentration using the Kodak Gel Logic 100 imaging system and associated software (Eastman Kodak, New Haven, CT, USA) Fig. 1.

RNA isolation and real-time RT-PCR of GFP transformants

Total RNA was isolated from mycelia collected by filtration (Skory 2002) from shake flask cultures of the respective isolates of each construct. The mycelia were disrupted using glass beads (Skory 2002) and the RNA purified using the RNeasy Total RNA kit (Qiagen) followed by DNase treatment with Turbo DNA-free (Ambion, Austin, TX, USA) for 30 min. RNA quality and quantity was confirmed using the 2100 Bioanalyzer Lab-on-Chip system (Agilent, Wilmington, DE, USA). Isolated RNA of similar quantity and quality was used as the template for one step real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to produce a 195 bp product using the Lightcycler RNA Master Hybridization Probes (Roche

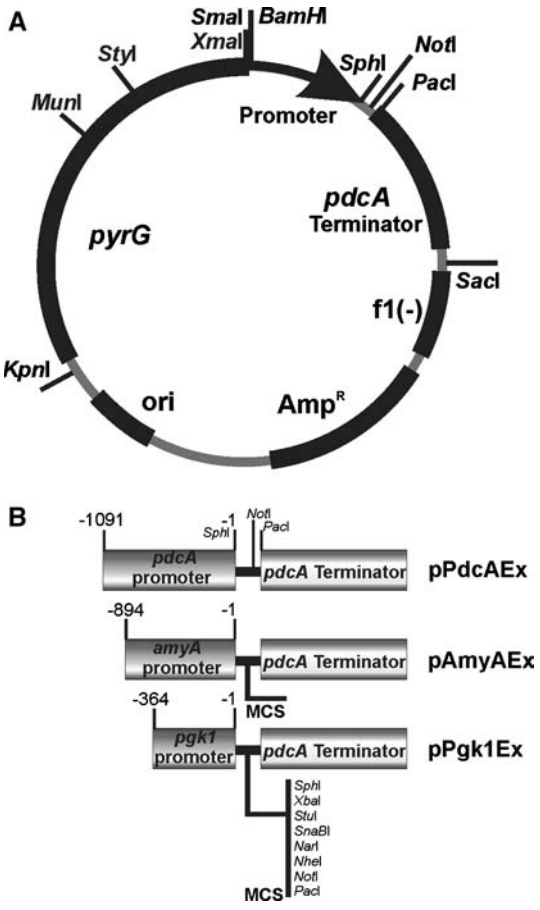

Fig. 1 Construction of the *R. oryzae* expression vectors. **a** Common feature positions of the expression plasmids. The *pdcA* terminator and placement of promoters is denoted. Amp^R is the ampicillin resistance gene; *pyrG* is the Omp decarboxylase gene for uracil complementation; ori is *E. coli* replication origin, f1(-) is origin for single stranded DNA. **b** The size and configuration of the promoters, cloning sites, and *pdcA* terminator placed in the expression plasmids

Applied Sciences, Indianapolis, IN, USA) mix along with the following gene-specific primers (Sigma Genosys, The Woodlands, TX, USA) and 5′ nuclease probes labeled with 6FAM and Black Hole Quencher I (Biosearch Technologies, Novato, CA, USA): GFP; GFP5Taq3–3, GFP3Taq3, and GFPprobe3 and 18S rRNA; 18STaq5, 18STaqAS, and 18Sprobe (Table 1). The qRT-PCR GFP assay, as well as no RT controls, were performed on the Roche Lightcycler using the following parameters: Reverse Transcription, 61°C 20 min, 1 cycle; 95°C 30 s, 1 cycle, and 45 amplification/detection cycles of 95°C, 5 s, annealing and detection at 60°C, 12 s and extension at 72°C, 30 s. A standard curve was created by performing RT-PCR with dilutions ranging from 10^9 to 10^3 copies of *in vitro* transcribed *gfp* RNA. The standard curve had a slope of –3.8 (greater than 100% efficiency), an r^2 value of 0.998, and a standard error of 0.106 cycles. There was no detectable signal from no-template control samples or reactions without reverse transcriptase. Samples were normalized to the 18S rRNA signal. All samples, standards, and controls were performed in triplicate.

Determination of gene copy number

The *gfp* gene copy number was determined using real-time PCR assays with total DNA as the template. DNA was obtained from the isolates via disruption of approximately 10 mg of mycelia using glass beads (Skory 2002) and the OmniPrep Genomic DNA Isolation kit (Geno Technology, St. Louis, MO, USA) according to manufacturer's instructions. The *gfp* real time PCR assay used the same primers, probe, and cycling condition used for RT-PCR. A real time PCR assay was developed for the single copy gene glucoamylase A, *amyA* (details of assay to be published elsewhere) and used to calibrate the copy number for GFP isolates. Signal was quantified by a standard curve and normalized to DNA quantity used in reactions. All samples, standards, and controls were performed in triplicate.

Results

Transformation of *R. oryzae* with *gfp* expression vectors

After biolistic transformation, several single spore isolates from transformations of each GFP expression construct were obtained for further analysis. The individual isolates were cultured for 72 h to ensure maximum protein expression, since preliminary experiments showed no loss of fluorescence over that detected at 24 and 48 h after inoculation. Mycelia were collected from the culture for microscopic visualization with white light and fluorescent illumination. As shown in Fig. 2a, all three constructs demonstrate fluorescence indicative of GFP expression, while untransformed *R. oryzae* pyr17 mycelia did not exhibit any auto-fluorescence with the filter sets used in this study (Fig. 2a-4). Based on fluorescent intensity, the pPdcA-*gfp* construct appeared to express the most GFP, while pPgk1-*gfp* appeared to be the lowest and the pAmyA-*gfp* construct intermediate (Fig. 2a). There were differences in intensity among various hyphal branches of the mycelium (data not shown). It was not just a matter of one particular mycelial structure (i.e. originating from a single spore) having more fluorescence. Instead, the different intensities seemed be associated with

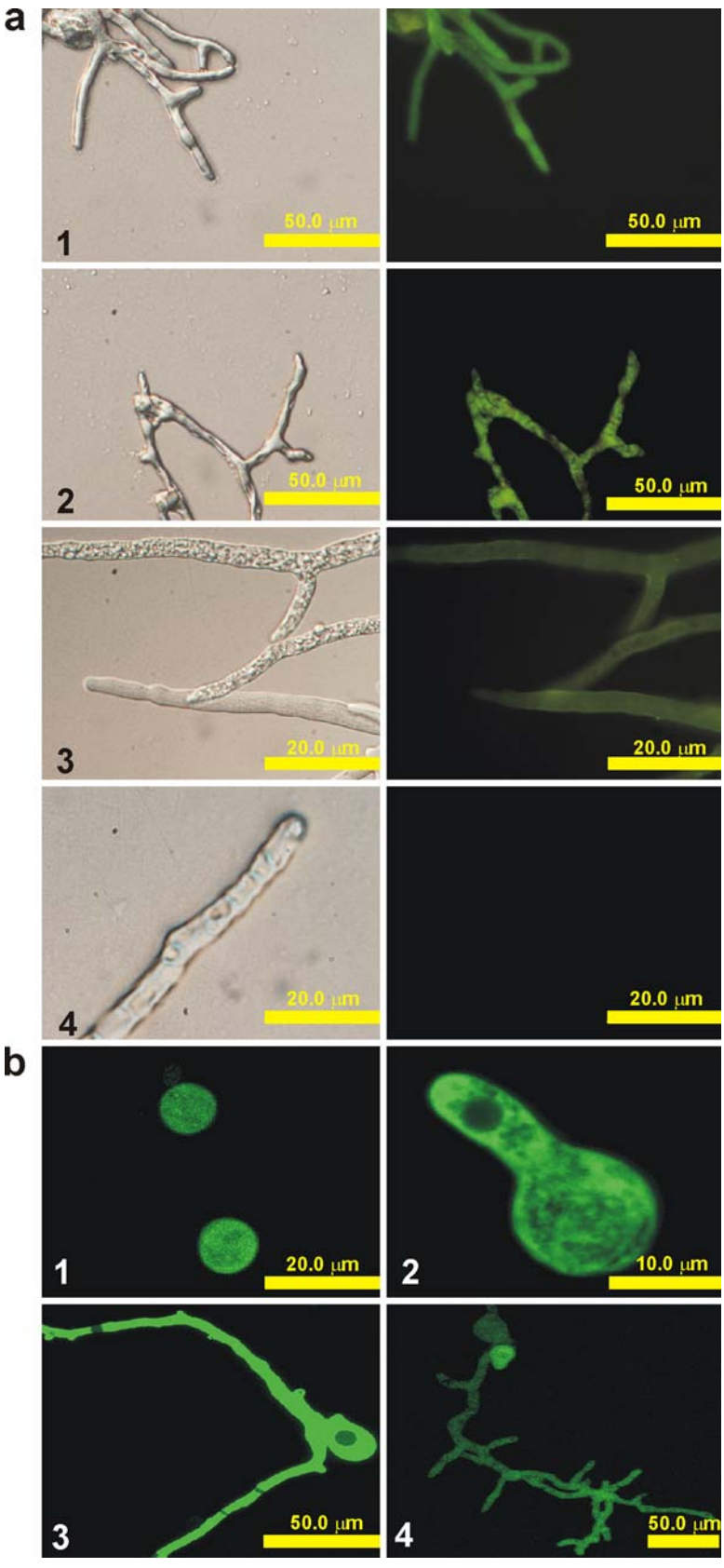

Fig. 2 **a** Light and fluorescent micrographs of the *R. oryzae* transformed with the *gfp* expression constructs. *1* pPdcA-*gfp* construct (×40), *2* pAmyA-*gfp* construct (×40), *3* pPgk1-*gfp* construct (×100), *4* untransformed *R.oryzae* pyr17 . **b** Confocal micrographs of *R. oryzae* transformed with the pPdcA-*gfp* construct. *1* Spores (×100), *2* germling (×200), *3* (×80) and *4* (×40) developing mycelia

unique branches of the structures. Within each branch, the fluorescence appears to be evenly distributed throughout the mycelia and not directed to any organelle. This aspect is more clearly seen in Fig. 2b, as GFP fluorescence is seen throughout the spores and mycelia in the confocal micrographs of a pPdcA-*gfp* transformant. Since *Rhizopus* is coenocytic and lacks cross walls, it is difficult to determine if production is occurring in a specific region of the hyphae and diffusing throughout the cell. Previous work with secreted GFP expression in *Aspergillus niger* suggests that most GFP accumulation is occurring at the growing hyphal tip where the cell is most metabolically active (Gordon et al. 2000). This phenomenon does not appear to be occurring here, since fluorescence is not any greater at the hyphal tips (Fig. 2). We believe this suggests that accumulated GFP remains stable throughout the hyphae, as it is similar to what has been previously reported for internal expression of GFP in other filamentous fungi (Lorang et al.2001).

To determine the quantity of the GFP accumulated in *R. oryzae*, we obtained crude protein extracts from mycelia grown in shake flask cultures for 72 h. GFP was not discernible in the cleared lysates on a Coomassie blue stained SDS-PAGE gel (data not shown), but could be detected on a western blot (Fig. 3). The western blot shown (Fig. 3) is representative of multiple experiments. Untransformed *R. oryzae* strains do not demonstrate a 27 kD band representative of the GFP protein (Fig. 3, Lane 1). The accumulation of GFP from the pPgk1-*gfp* construct appears to be the lowest of the three constructs with a faint band visible for one of the samples (Fig. 3, Lane 3) and one sample too low to allow a discernable band to be visible in the captured image (Fig. 3, Lane 2). The pAmyA-*gfp* construct (Fig. 3, Lanes 6 and 7) bands give a roughly similar result with transformant seven (Fig. 3, Lane 7) demonstrating a band that is comparatively stronger than the pPgk-*gfp* transformant (Fig. 3, Lane 3) both visually and with protein loaded taken into account. Accumulation of GFP from the pPdcA-*gfp* construct (Fig. 3, Lanes 4 and 5) appears at least fivefold stronger than the pAmyA-*gfp* construct under the growth conditions used here. The GFP protein does not appear to be subject to proteolytic degradation, since we do not see any detectable degradation products in the western blot. The accumulation of GFP, as determined in the western blot, generally follows the fluorescent intensity seen in the mycelial micrographs; i.e., pPdcA-*gfp* > pAmyA-*gfp* > pPgk1-*gfp*. Overall, by comparing GFP accumulation to a purified standard using relative densitometry, we were able to estimate the expression of GFP amounts to roughly 30 ng GFP/mg of soluble protein in the pPdcA-*gfp* isolates and approximately 4 ng/mg protein and 2.5 ng/mg protein for the pAmyA-*gfp* and pPgk1-*gfp* isolates, respectively.

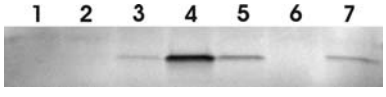

Fig. 3 Western blot representative crude extracts of *R. oryzae* transformed with *gfp* expression constructs. *Lane 1* Untransformed *R. oryzae* NRRL 395 (28 μg), *Lane 2* pPgk1-*gfp* isolate #8 (19.3 μg), *Lane 3* pPgk1-*gfp* isolate #3 (25.4 μg), *Lane 4* pPdcA-*gfp* isolate #8 (28 μg), *Lane 5* pPdcA-*gfp* isolate #6 (21.5 μg), *Lane 6* pAmyA-*gfp* isolate #5 (18 μg), *Lane 7* pAmyA-*gfp* isolate #7 (32.8 μg). *Numbers in parenthesis* indicate total protein loaded for each sample

Transcript level and gene copy number of *gfp*

In order to determine if the protein expression levels seen in the mycelia correlated to transcript accumulation in the isolates, real time qRT-PCR was performed using total RNA isolated from pPdcA-*gfp*, pPgk1-*gfp*, and pAmyA-*gfp* cultures. The transcript levels determined by RT-PCR, calculated from a standard curve linear from 10^9 to 10^3 copies, correlated with the apparent fluorescent intensity of the mycelia and protein detected in the western blot (Fig. 4). A northern blot was also performed (data not shown) to ensure the presence of full length transcript and provide further validation to the qRT-PCR results. Again, the pPdcA-*gfp* construct expressed the highest level with an average among nine isolates of 1.63×10^5 transcripts/ng total RNA, while the pPgk1-*gfp* was lowest at 1.38×10^3 transcripts and pAmyA-*gfp* at 2.44×10^4 transcripts (Fig. 4).

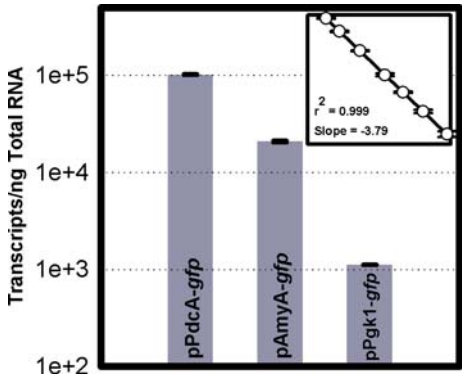

Fig. 4 Transcriptional analysis of *gfp* constructs. Real-Time RT-PCR performed as described in Materials and methods with samples normalized to 18S rRNA. Error bars indicate standard error among nine isolates for each promoter construct. *Inset* graph is the *gfp* RNA standard curve using concentrations from 10^9 to 10^3 copies

We also determined whether the transcription levels were impacted by gene copy number. While we found the copy number ranged from 2 to in excess of 100 copies in isolates of each of the three constructs (Table 2), it had no obvious impact on the overall average transcript level seen for the three *gfp* constructs (Fig. 4) and accounted for only minor differences within the individual constructs.

Discussion

With the goal of this work to determine a baseline expression level and the capacity of *R. oryzae* to express heterologous proteins, we chose three different promoters with unique regulation. The *pgk1* and glucoamylase promoters have been used previously in Mucorales expression systems (Yanai et al.1991; Takaya et al. 1994, 1995; Houghton Larsen and Pedersen 2003), while the *pdcA* promoter has not. The *pdcA* promoter of *R. oryzae* is reported to be transcribed with glucose as the carbon source, but is not active with glycerol (Skory 2003). In contrast, the *pgk1* promoter of *R. oryzae* is expressed with most fermentative carbon sources (Gao and Skeen 2002), but weakly expressed with glycerol as the energy source (Takaya et al. 1994). The *amyA* promoter is highly expressed when cellobiose or soluble starch is used as a carbon source, moderately in the presence of glucose, and weakly on glycerol and maltose (J. Mertens, unpublished results).

Outside of these reports, there is little information delineating the actual sequence that defines the promoter region or potential regulatory elements of the three promoter elements used here. Previous work with the *R. niveus pgk*2 promoter demonstrated little difference in expression of β-glucuronidase with promoter constructs longer than 360 bp (Takaya 1995), leading to the use of a 365 bp promoter fragment to drive expression of GFP in our system. The glucoamylase promoter has also been used previously in other filamentous fungus with a rat adenosine A1 receptor expressed at low levels in *M. circinelloides* using a translational fusion with the *glaM* gene that contained a 1.9 kb *glaM* promoter region (Houghton-Larsen and Pederson 2003). In addition, a *R. oryzae* 600 bp *amyA* promoter fragment has been used to express blasticidin S in *R. niveus* (Yanai et al. 1991). However, to date characterization of this glucoamylase promoter and potential regulatory regions within the promoter region have yet to be reported. With no a priori knowledge of the *amyA* promoter region, an 894 bp *amyA* promoter fragment was used in an effort to capture all potentially important regulatory elements. A similar strategy was used in selecting the 1.091 kb *pdcA* promoter fragment. The size of the promoter region used for expression certainly has the potential to impact expression level, but without clear definition of potential regulatory elements, the size of the promoter element used becomes largely arbitrary. Clearly, additional work will be required to gain a more complete definition of the promoter regions used here. However, the promoter fragments used in previous studies and in our work do provide a foundation that can be used to systematically improve expression of heterologous proteins using the different promoters.

Rhizopus oryzae isolates transformed with the three different promoter constructs demonstrated fluorescence attributable to GFP (Fig. 2a). While a major proportion of the mycelia exhibit fluorescence, some branches of the hyphae show only weak fluorescence (data not shown). When GFP was expressed in *Absidia glauca*, a similar result was seen and was attributed to heterokaryosis or more specifically the segregation of genetically distinct nuclei to different branches of the mycelium (Schilde et al. 2001). It is unknown to what extent this may possibly occur with our plasmid. The *pyrG* gene on each plasmid would be required for synthesis of hyphal growth, yet it is feasible that enough diffusion of synthesized uracil could occur through the hyphae to allow continued replication of nuclei lacking the selectable plasmid. Alternatively, this may just represent differences in plasmid copy number along distinct branches of the mycelium as copy number is expected to be variable along the mycelial branches due to mitotic instability.

To gain a clearer understanding of the *R. oryzae* system, we performed transcriptional analysis to determine the effects of transcript level on protein production. The transcript level (Fig. 4) clearly paralleled the amount of accumulated protein and generally correlated with the chosen promoter. As mentioned earlier, differences in the promoter lengths could potentially have an impact on overall transcript levels. Without any defined knowledge of the promoters used here, we cannot make any definitive judgements on actual promoter strength. However, it should be noted that the *pdc* gene was recently shown to be one of the most highly expressed genes in *Aspergillus oryzae* (Maeda et al. 2004).

It has also been reported that transcript level of heterologous genes is lower relative to homologous genes in some cases, suggesting problems with transcript stability (Gouka et al. 1996, 1997; Paloheimo et al. 2003); this does not appear to be the case here. The amount of accumulated *gfp* transcript produced by

the pAmyA-*gfp* construct is essentially the same as the transcript level seen with the native *amyA* gene when cultures were grown under the same conditions used here (J. Mertens, unpublished results). It would also seem from the pAmyA-*gfp* transcription results that the *pdcA* terminator does not have a negative impact on transcription or transcript stability. Based on the work presented here, we cannot rule out any impact on translation efficiency when the *pdcA* terminator is used in combination with any of the described promoters.

Gene copy number appears to have little impact on expression as there were only minor differences among a number of isolates of each expression construct (Table 2). This result is similar to what has been seen in some cases with *Aspergillus* sp. (Radzio and Kück 1997), but very different from *Trichoderma reesi* (Karhunan et al. 1993) and some of the yeast systems where very high copy numbers lead to a reduction in protein expression (Parekh et al. 1995; Hohenblum et al. 2004). In *R. niveus*, copy number did have some effect, as higher copy number generally resulted in more protein expression (Takaya 1994). However, it appeared that once the copy number reaches a certain level, expression either stays roughly the same or declines.

Table 2 *gfp* gene copy number and transcript/copy from *R. oryzae* transformants

Isolate	*gfp* copy number	Transcripts/copy number
pPdcA-*gfp* #1	47	2.86E+02
pPdcA-*gfp* #2	2	2.39E+04
pPdcA-*gfp* #3	37	3.46E+03
pPdcA-*gfp* #4	60	5.50E+03
pPdcA-*gfp* #5	40	3.09E+03
pPdcA-*gfp* #6	103	3.05E+03
pPdcA-*gfp* #7	6	2.81E+03
pPdcA-*gfp* #8	49	3.70E+03
pPdcA-*gfp* #9	116	2.84E+03
pAmyA-*gfp* #1	45	1.69E+03
pAmyA-*gfp* #2	6	1.28E+02
pAmyA-*gfp* #3	57	1.45E+01
pAmyA-*gfp* #4	85	6.02E+01
pAmyA-*gfp* #5	16	1.83E+03
pAmyA-*gfp* #6	20	4.14E+01
pAmyA-*gfp* #7	14	3.29E+03
pAmyA-*gfp* #8	11	1.48E+02
pPgk1-*gfp* #1	49	6.52E+01
pPgk1-*gfp* #2	12	2.55E+01
pPgk1-*gfp* #3	5	3.96E+02
pPgk1-*gfp* #4	10	1.96E+02
pPgk1-*gfp* #5	13	1.25E+02
pPgk1-*gfp* #6	31	2.23E+01
pPgk1-*gfp* #7	32	3.29E+01
pPgk1-*gfp* #8	45	1.29E+01
pPgk1-*gfp* #9	14	8.01E+01

It should be recognized that plasmid copy number and transcript level was determined as a representative average of the mycelial population as was the transcript level. It is fully expected that there will be some change in copy number among the nuclei of the mycelial branches due to mitotic instability in this coenocytic fungus, even in the presence of selection pressure. In fact we see some evidence for this in the spatial distribution of the GFP. Mitotically stable replication of the multi-copy concatemers in *Rhizopus* would clearly be preferable and eliminate the need for constant selection pressure. However, autonomously replicating plasmids in yeast or *E. coli* suffer a similar fate. Furthermore, gene copy number appears to have little or no impact on overall gene expression in our system, suggesting that single or low-copy gene integration would be just as effective for overall expression. In this regard, there has been some recent success in integrating transformed DNA in *R. oryzae* using linearized plasmid DNA (Skory 2002) or *Agrobacterium*-mediated transformation (Michielse et al. 2004). While there is further need for improvement of these methods, these advances have the potential to favorably position *R. oryzae* for use as a heterologous protein expression system.

This work represents the first report of heterologous protein expression using modified promoters in *R. oryzae*. We have successfully expressed GFP using three different expression constructs, paving the way for continued systematic improvements in the development of the *R. oryzae* expression system. We have also shown that transcript level is high and appears to be stable regardless of gene copy number. We expect through a continued systematic process of addressing the problems identified in this and other filamentous fungal systems that heterologous protein production will increase, potentially making this expression system more robust and amenable to industrial use.

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